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Effects of PS1 Deficiency on Membrane Protein Trafficking in Neurons

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Summary

We have examined the trafficking and metabolism of the β-amyloid precursor protein (APP), an APP homolog (APLP1), and TrkB in neurons that lack PS1. We report that PS1-deficient neurons fail to secrete Aβ, and that the rate of appearance of soluble APP derivatives in the conditioned medium is increased. Remarkably, carboxyl-terminal fragments (CTFs) derived from APP and APLP1 accumulate in PS1-deficient neurons. Hence, PS1 plays a role in promoting intramembrane cleavage and/or degradation of membrane-bound CTFs. Moreover, the maturation of TrkB and BDNF-inducible TrkB autophosphorylation is severely compromised in neurons lacking PS1. We conclude that PS1 plays an essential role in modulating trafficking and metabolism of a selected set of membrane and secretory proteins in neurons.

Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disorder of late life, is characterized by the deposition of 42–43 amino acid Aβ peptides and neurofibrillary tangles in the hippocampus and cerebral cortex of affected individuals. The N and C termini of Aβ peptides are generated following endoproteolytic cleavage of larger type 1 integral membrane β-amyloid precursor proteins (APP) by "β-" and "γ-secretase" activities, respectively (reviewed in Selkoe, 1997).

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Early-onset familial forms of AD are caused by autosomal dominant inheritance of mutations in genes encoding APP (reviewed in Selkoe, 1997), presenilin 1 (PS1), and presenilin 2 (PS2) (reviewed in Hardy, 1997). PS1, a polytopic membrane protein (Doan et al., 1996; Li and Greenwald, 1998), is structurally and functionally homologous to SEL-12, a *C. elegans* protein that facilitates Notch/lin-12 signaling (Levitan and Greenwald, 1995). Mice with ablated PS1 alleles ($PS1^{-/-}$ mice) die late in embryogenesis and exhibit abnormalities in somite segmentation and loss of segment polarity that lead to patterning defects in axial skeleton and spinal ganglia, and hemorrhages in the CNS (Shen et al., 1997; Wong et al., 1997). Very recently, De Strooper and colleagues (1998) documented that primary neuronal cultures generated from $PS1^{-/-}$ mouse embryos fail to secrete Aβ peptides, coincident with intracellular accumulations of APP C-terminal fragments (CTFs) containing varying segments of the Aβ sequence. These observations were interpreted to suggest that PS1 influences intramembranous γ-secretase processing of APP. Despite the novelty of these latter conclusions, little information was available that directly assessed the specificity of PS1 deficiency.

In the present manuscript, we examine the metabolism and trafficking of a variety of membrane and secretory proteins in primary neurons lacking PS1. Confirming the studies by De Strooper and coworkers (1998), we demonstrate that PS1-deficient neurons fail to secrete Aβ. In contrast to the studies by De Strooper and colleagues, however, we document that the rate of appearance of soluble APP ($APP^{s\alpha}$) derivatives in medium of PS1-deficient neurons is quantitatively increased. Remarkably, a CTF derived from amyloid precursor-like protein 1 (APLP1), also accumulates in PS1-deficient neurons; in view of the limited homology between the APP and APLP1 transmembrane domains, we conclude that PS1 has a much broader role in promoting intramembrane cleavage and/or degradation of CTFs derived from APP, its homologs, and perhaps other transmembrane polypeptides. Moreover, we now document that the rate of acquisition and steady-state levels of complex oligosaccharide modifications of TrkB, a tyrosine kinase receptor for brain-derived neurotrophic factor (BDNF), is diminished in PS1-deficient neurons. Consistent with these observations, BDNF-mediated TrkB autophosphorylation is severely compromised in PS1-deficient neurons. Interestingly, and with very few exceptions, the rate and pattern of newly synthesized proteins that appear on the plasma membrane and conditioned medium of wt and PS1-deficient neurons are remarkably similar. These results support the view that loss of PS1 activity has a highly selective role in the trafficking and turnover of selected membrane proteins in neurons.

Results

Elimination of PS1 Abrogates Aβ Secretion

Recent studies by De Strooper and colleagues provided compelling evidence that primary neurons from $PS1^{-/-}$

mouse embryos exhibit marked reductions in secretion of Aβ peptides, coincident with the intracellular accumulation of a complex set of APP C-terminal fragments (CTFs) containing Aβ peptide–related sequences (1998). We prepared cortical neuronal cultures from day 15.5 p.c. PS1$^{-/-}$, PS1$^{+/+}$, or PS1$^{+/-}$ embryos and assayed steady-state levels of APP metabolites after 5 days. At this point, surviving neurons appeared morphologically differentiated, and nonneuronal cells represented <5% of all cells in the culture (data not shown). Western blot analysis of detergent lysates prepared from neuronal cultures using CT15, an antibody specific for the carboxyl-terminal 15 amino acids of APP (Sisodia et al., 1993; Zheng et al., 1995), revealed that APP CTFs were markedly elevated in PS1$^{-/-}$ cultures (Figure 1A, lanes 3 and 4), compared to sister cultures prepared from littermates with PS1$^{+/+}$ or PS1$^{+/-}$ genotypes (Figure 1A, lanes 1 and 2, respectively), despite equivalent steady-state levels of ~100–120 kDa, full-length APP-695 between samples. In addition, quantitative sandwich ELISA analyses revealed that in conditioned medium of PS1$^{-/-}$ neurons, Aβ-related peptides terminating at residue 40 or 42 (x-40 or x-42, respectively) were virtually eliminated (Figure 1B). Metabolic labeling of neuronal cultures with ^{35}S-methionine for 5 hr and immunoprecipitation analysis of detergent lysates with CT15 antibody confirmed the Western blot analyses; while levels of full-length radiolabeled APP in PS1$^{+/-}$ and PS1$^{-/-}$ cultures were comparable (Figure 1C, lanes 1 and 2, respectively), radiolabeled APP CTFs accumulated in PS1$^{-/-}$ cells (Figure 1C, lane 2). Moreover, immunoprecipitation analysis using Mab4G8 (Kim et al., 1990), specific for epitopes between residues 17–28 of Aβ, revealed that radiolabeled ~4 kDa Aβ and ~3–3.5 kDa Aβ-related peptides, normally secreted into medium of PS1$^{+/-}$ cells, failed to accumulate in the conditioned medium of PS1$^{-/-}$ cells (Figure 1C, compare lanes 3 and 4, respectively).

To demonstrate that the accumulation of APP CTFs in PS1$^{-/-}$ cells is solely a consequence of the loss of PS1, we examined the steady-state levels of APP metabolites in detergent extracts prepared from brains of day 15.5 p.c. PS1$^{+/+}$, PS1$^{-/-}$, and PS1$^{-/-}$ mice rescued by human PS1 transgenes; we (Davis et al., 1998) and others (Qian et al., 1998) previously documented that the developmental deficits in PS1$^{-/-}$ mice are efficiently rescued by expression of transgenes encoding either human PS1 or the FAD-linked, A246E human PS1 variant. Similar to the results obtained by analysis of primary neuronal cultures, we observed a significant elevation in levels of ~8.5–12 kDa CTFs in lysates of PS1$^{-/-}$ brain (Figure 2A, lane 4; compare to levels in PS1$^{+/+}$, lane 3). Importantly, the APP CTFs exhibit electrophoretic migration similar, if not identical, to CTFs that accumulate in mouse brain (Figure 2A, lane 1) or in the brain of mice that overexpresses a mouse APP-695 transgene (D. R. B., unpublished data) (Figure 2A, lane 2). Hence, it is highly likely that the mouse APP CTFs which accumulate in PS1$^{-/-}$ brain are generated by constitutive activities rather than alternative or aberrant proteolytic events. Moreover, the APP CTFs in brains of PS1$^{-/-}$ mice are almost completely restored to wild-type levels upon expression of human transgenes encoding either human PS1 or the A246E human PS1 variant (Figure 2A, compare lane 3 to lanes 5 and 6, respectively). Western blot analysis of the brains of rescued PS1$^{-/-}$ mice using αPS1loop, an antibody specific for PS1 epitopes between amino acids 300–360 (Thinakaran et al., 1996), revealed the presence of a C-terminal PS1 fragment,

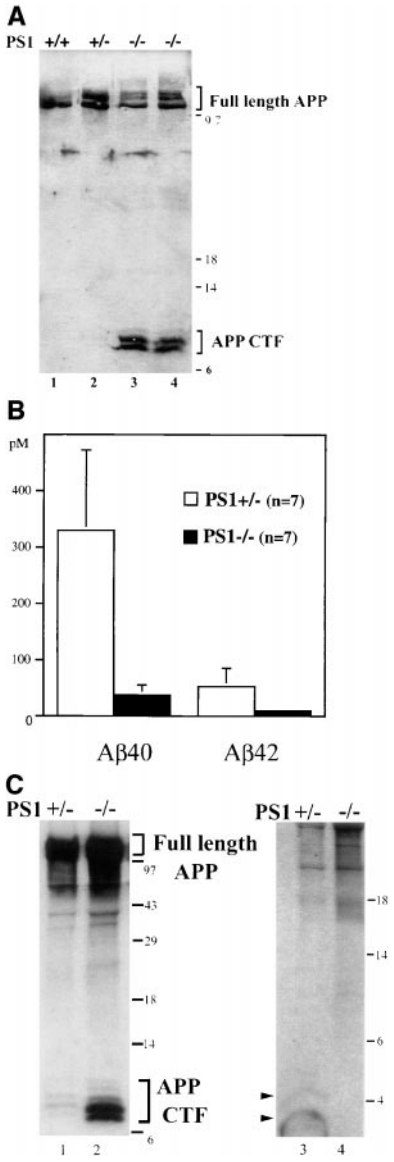

Figure 1. Accumulation of APP C-Terminal Fragments and Reduced Secretion of Aβ Peptides from PS1$^{-/-}$ Neurons

(A) Detergent lysates of PS1$^{+/+}$ (lane 1), PS1$^{+/-}$ (lane 2), and PS1$^{-/-}$ (lanes 3 and 4) neuronal cell cultures were immunoblotted using CT15, an antisera recognizing the C terminus of APP. The positions of the full-length APP and the C-terminal fragments (APP CTF) are marked by brackets.

(B) Determination of Aβx-40 and Aβx-42 levels from conditioned medium of PS1$^{+/-}$ and PS1$^{-/-}$ neuronal cultures. The concentrations of Ab peptides for each genotype are plotted (in pM) as mean ± standard deviation (n = 7).

(C) Detergent lysates or conditioned media from PS1$^{+/-}$ (lanes 1 and 3) and PS1$^{-/-}$ (lanes 2 and 4) neuronal cell cultures radiolabeled with ^{35}S-methionine were immunoprecipitated with CT15 (lanes 1 and 2) or with 4G8, an antisera specific for Ab peptides (lanes 3 and 4), respectively. Full-length APP and APP CTF are indicated by brackets, and Ab and p3 peptides are marked by arrowheads.

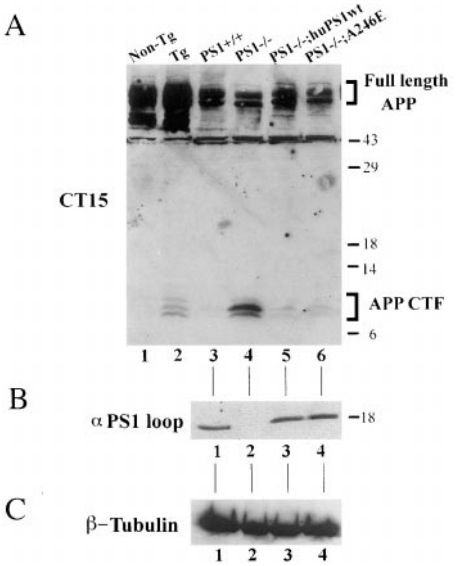

Figure 2. APP CTF Are Restored to Normal Levels in PS1$^{-/-}$ Rescued Embryos

(A) Total proteins (30 μg) extracted from brains of 2-month-old control (lane 1) and transgenic mouse expressing APP transgene (lane 2) or E15.5 control (lane 3), PS1$^{-/-}$ (lane 4), PS1$^{-/-}$;huPS1wt (lane 5), and PS1$^{-/-}$;A246E (lane 6) embryos were analyzed by immunoblotting using APP-specific antibody CT15. Full-length APP and APP CTF are indicated.

(B) Protein extracts from E15.5 control (lane 3), PS1$^{-/-}$ (lane 4), PS1$^{-/-}$;huPS1wt (lane 5), and PS1-/-;A246E (lane 6) brain were subjected to immunoblotting with a PS1-specific antibody, αPS1Loop, specific for epitopes in the "loop" domain of mouse and human PS1.

(C) The blot in (B) was stripped, then reprobed with a β-tubulin-specific monoclonal antibody.

generated by endoproteolytic cleavage of transgene-encoded human PS1 (Figure 2B, lanes 3 and 4, respectively).

PS1 Exerts Multiple Effects on APP Metabolism

The highly reproducible observation that PS1-deficient neurons fail to secrete Aβ peptides and accumulate APP CTFs (De Strooper et al., 1998; Figure 1, above) left open the possibility that loss of PS1 activity might have more widespread influences on APP metabolism. In this regard, De Strooper and colleagues reported that neuronal secretion of C-terminally truncated APP derivatives, termed APP$^{s\alpha}$ and APP$^{s\beta}$, generated by endoproteolytic cleavage by α- or β-secretase(s), respectively, are not influenced by the loss of PS1. To corroborate these findings, we examined the maturation and processing of APP in PS1$^{+/-}$ and PS1$^{-/-}$ primary neurons following infection with a recombinant adenovirus harboring cDNA encoding wild-type human APP695. Cells were infected for 3 days, pulse-labeled in medium containing ^{35}S-methionine for 1 hr, then chased for either 2 or 4 hr in medium containing unlabeled L-methionine. APP-related polypeptides were immunoprecipitated from detergent lysates using CT-15 antibody. While full-length ~100–125 kDa APP and ~9–12 kDa APP CTFs accumulated to similar levels in PS1$^{+/-}$ and PS1$^{-/-}$ cells after

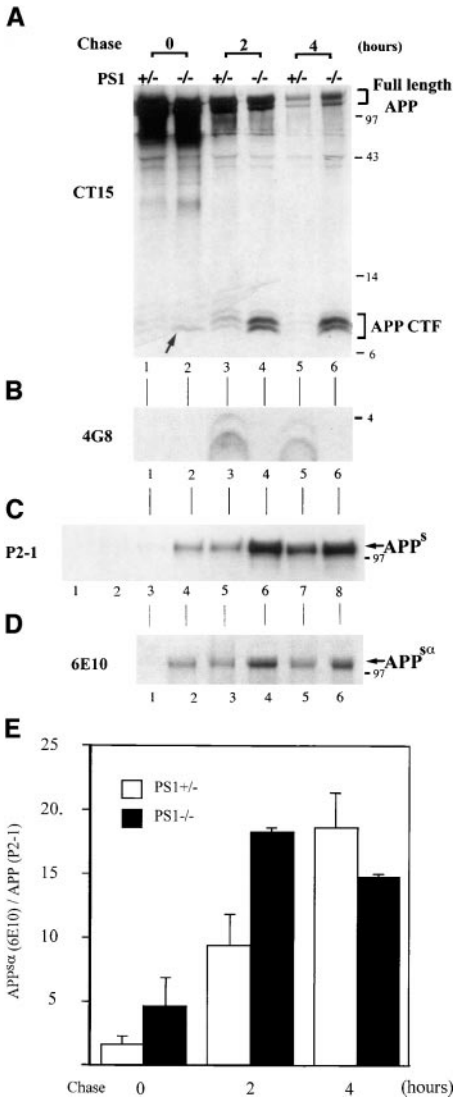

Figure 3. Altered APP Metabolism in PS1$^{-/-}$ Neurons

(A) Following 3 days of infection with adenovirus expressing human APP, PS1$^{+/-}$ (lanes 1, 3, and 5) and PS1$^{-/-}$ (lanes 2, 4, and 6) neuronal cultures were pulse-labeled for 1 hr (lanes 1 and 2) with ^{35}S-methionine, then chased in the presence of L-methionine for 2 hr (lanes 3 and 4) and 4 hr (lanes 5 and 6). Full-length APP and CTF of APP were immunoprecipitated with CT15. Arrow indicates an ~8.5 kDa CTF.

(B) Ab and p3 peptides; (C) soluble APP derivatives (APP^{s}) or (D) α-secretase-generated APP^{s} (APP$^{s\alpha}$) were immunoprecipitated with 4G8, P2-1, or 6E10 antisera, respectively, from conditioned media of the corresponding neuronal cultures as shown in (A). Lanes 1 and 2 of (C) were from conditioned media of wt neuronal cultures labeled for 5 hr with ^{35}S-methionine.

(E) Quantitative analysis of APP$^{s\alpha}$ release. Experiments were performed in triplicate on different days. The APP$^{s\alpha}$ signals at each point of the pulse-chase experiments were quantified by phosphoimaging. Error bar is SEM.

the 1 hr labeling period (Figure 3A, lanes 1 and 2, respectively), the level of an ~8.5 kDa polypeptide was clearly elevated in PS1$^{-/-}$ cells (Figure 3A, lane 2). While the identity of this fragment has not been established, it likely represents the membrane-retained APP CTF generated by α-secretase cleavage of APP (see below, Figure 3D). After 2 hr of chase, the ~8.5 kDa and ~9–10

kDa APP CTFs, presumably generated after cleavage at or near Aβ +11 (De Strooper et al., 1995; Simons et al., 1996), accumulate to markedly higher levels in PS1$^{-/-}$ cultures compared to CTFs in PS1$^{+/-}$ cultures (Figure 3A, compare lane 4 with lane 3). After 4 hr of chase, APP CTFs in PS1$^{+/-}$ cultures were nearly eliminated, while in PS1$^{-/-}$ cultures, these fragments were stabilized (Figure 3A, lanes 5 and 6, respectively). Finally, and supporting earlier steady-state experiments (Figure 1B, 1C), Aβ-related peptides fail to be secreted by PS1$^{-/-}$ cultures (Figure 3B, lanes 4 and 6; compare to Aβ peptides secreted by PS1$^{+/-}$ cultures, lanes 3 and 5).

Our observation that the levels of an APP C-terminal fragment of ~8.5 kDa are increased in lysates prepared from PS1$^{-/-}$ cultures immediately after pulse labeling (Figure 3A, lane 2) suggested that the rate of α-secretase processing of APP might be influenced by the loss of PS1. To clarify this issue, we first immunoprecipitated radiolabeled soluble APP derivatives (APP^{s}) with MabP2-1, a monoclonal antibody specific for an epitope in the human APP ectodomain (Van Nostrand et al., 1989). As expected, P2-1 failed to detect mouse APP^{s} in the conditioned medium of uninfected PS1$^{+/-}$ or PS1$^{-/-}$ cultures (Figure 3C, lanes 1 and 2, respectively). However, and most surprisingly, an ~110 kDa human APP^{s} was elevated in the conditioned medium of PS1$^{-/-}$ cultures immediately after the pulse-labeling period (Figure 3C, compare lane 4 to PS1$^{+/-}$ medium in lane 3), and this difference is still apparent after 2 hr in chase medium (Figure 3C, compare lane 6 to PS1$^{+/-}$ medium in lane 5). After 4 hr, the levels of APP^{s} in the medium of PS1$^{+/-}$ and PS1$^{-/-}$ cultures appear similar (Figure 3C, compare lanes 7 and 8, respectively). To quantitatively assess the levels of α-secretase (APP^{sα})-generated soluble derivatives, we subjected the conditioned medium of PS1$^{+/-}$ or PS1$^{-/-}$ cultures to immunoprecipitation with Mab6E10, a monoclonal antibody that selectively binds a sequence in the human Aβ peptide domain (Kim et al., 1990; Thinakaran et al., 1996). Phosphorimaging analysis (Figure 3E) revealed that at the end of the 1 hr pulse-labeling period, APP^{sα} in medium of PS1$^{-/-}$ cultures are twice the levels as that observed in medium of PS1$^{+/-}$ cultures (Figure 3D, compare lanes 1 and 2, respectively; Figure 3E). Thus, the elevated levels of APP^{sα} in medium, coincident with increase in levels of an intracellular APP C-terminal fragment of ~8.5 kDa (Figure 3A, lane 2) in PS1$^{-/-}$ cultures immediately after pulse labeling, lead us to suggest that loss of PS1 expression enhances the rate of APP trafficking to the plasma membrane, the preponderant site for α-secretase cleavage of APP (reviewed in Selkoe, 1997). The 2-fold difference in accumulated APP^{sα} in medium between PS1-deficient and PS1$^{+/-}$ neurons persists over the initial 2 hr chase period (Figure 3D, compare lanes 3 and 4, quantified in Figure 3E). After 4 hr of chase, APP^{sα} in medium of PS1$^{-/-}$ cultures appears to plateau, while the levels of APP^{sα} in medium of PS1$^{+/-}$ cultures now appear comparable to that in medium of PS1-deficient cultures (Figure 3D, compare lanes 5 and 6; quantified in Figure 3E). Our interpretation of these findings is that the total pool size of α-secretase substrates in PS1$^{+/-}$ or PS1$^{-/-}$ cells is not substantially different. Rather, in PS1$^{-/-}$ cells, the burst of APP^{s} release corresponds to a pool of newly synthesized APP^{s} that rapidly transit the secretory apparatus and encounter α-secretase at or near the plasma membrane.

PS1 Influences Metabolism of APLP1

One interpretation of the observation that loss of PS1 leads to accumulation of APP C-terminal fragments and inhibition of Aβ production (De Strooper et al., 1998; Figures 2 and 3, above) is that PS1 selectively facilitates APP transmembrane domain cleavage by γ-secretase. To test whether endoproteolysis of additional membrane proteins might be compromised by the loss of PS1, we analyzed the metabolism of APLP1. APLP1, an APP homolog, is uniquely expressed in the nervous system (Wasco et al., 1992; von Koch et al., 1998). Although APP and APLP1 share significant regions of homology in the ectodomain and cytosolic domains, the homology between the APP and APLP1 transmembrane domains is limited, particularly at the APP "γ-secretase" sites (arrows) (Figure 4A). We stripped, then reprobed the Western blot of embryonic brain extracts shown in Figure 2A with CT11, a polyclonal antisera specific for epitopes in the C terminus of APLP1 (von Koch et al., 1998). While the steady-state levels of full-length ~90–120 kDa APLP1 were identical between samples, an ~7.5 kDa APLP1 C-terminal fragment was elevated in the PS1$^{-/-}$ brain extract (Figure 4B, lane 2). Moreover, and highly reminiscent of the results shown in Figure 2A, expression of transgene-encoded wild-type or A246E human PS1 variants in a PS1$^{-/-}$ background restored the APLP1 C-terminal fragment to levels normally observed in PS1$^{+/+}$ animals (Figure 4B, compare lanes 3 and 4 with lane 1). Similarly, immunoprecipitation analysis confirmed that an ^{35}S-methionine-labeled APLP1 C-terminal fragment accumulated to significant levels in PS1$^{-/-}$ primary neuronal cultures (Figure 4C, lane 2). To examine the biosynthesis and turnover of APLP1 C-terminal fragments, we immunoprecipitated APLP1-related polypeptides from the same detergent extracts of metabolically labeled cultures used in Figure 3A. While the levels of ~7.5 kDa APLP1 C-terminal fragments are comparable between PS1$^{+/-}$ and PS1$^{-/-}$ cultures at the end of the 1 hr labeling period (Figure 4C, compare lanes 3 and 4, respectively), it is readily apparent that relative to PS1$^{+/-}$ cultures, the APLP1 CTF in PS1$^{-/-}$ cultures is stabilized over the chase period (Figure 4C, compare lanes 6 and 8 to 5 and 7). These observations, taken together with the earlier demonstration that APP CTFs accumulate in PS1-deficient cells, argue in support of the view that loss of PS1 has a more general effect on the turnover of membrane-bound "stubs" derived from transmembrane proteins.

Diminished Functional TrkB Receptors in PS1-Deficient Neurons

Convinced that loss of PS1 activity had multiple effects on the metabolism of APP and APLP1 and their derivatives, we asked whether the metabolism of other membrane glycoproteins might also be altered in PS1-deficient neurons. For these studies, we chose to examine the maturation of TrkB, a tyrosine kinase receptor specific for brain-derived neurotrophic factor (BDNF) (Chao, 1992). PS1$^{+/-}$ and PS1$^{-/-}$ cultures were pulse-labeled

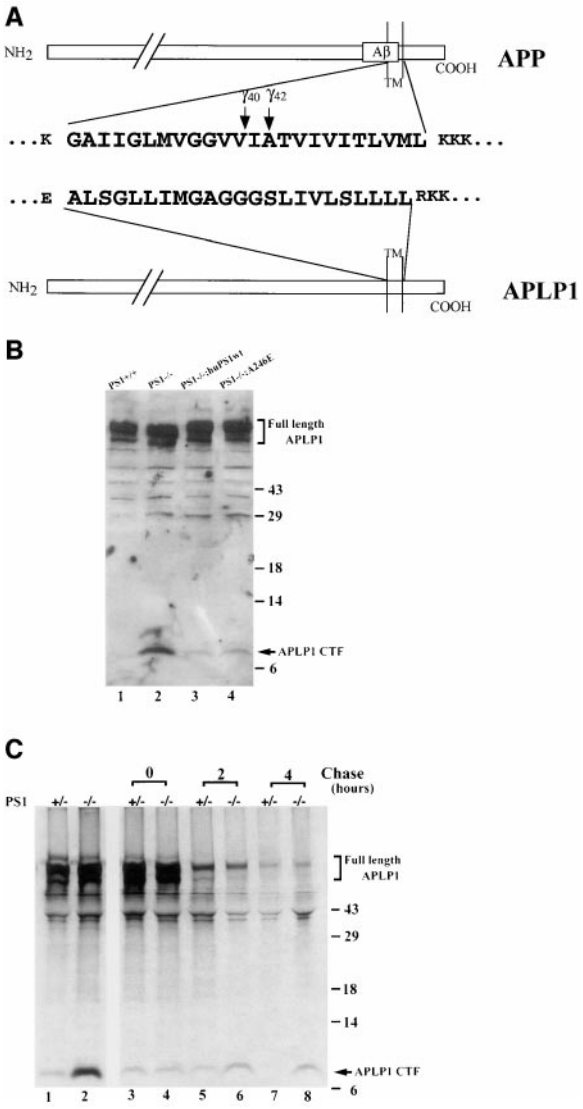

Figure 4. Accumulation of APLP1 CTF in PS1$^{-/-}$ Brain and Neuronal Cultures

(A) Comparison of the amino acid sequences of the transmembrane domains of APP and APLP1. Arrows denote γ-secretase cleavage sites of APP.

(B) Total protein extracts (30 μg) from E15.5 brains of PS1$^{+/+}$ (lane 1), PS1$^{-/-}$ (lane 2), PS1$^{-/-}$;huPS1wt (lane 3), and PS1$^{-/-}$;A246E (lane 4) mice were analyzed by immunoblotting with an APLP1 specific antibody, CT11. Arrow indicates a C-terminal fragment of APLP1.

(C) PS1$^{+/-}$ (lanes 1, 3, 5, and 7) and PS1$^{-/-}$ (lanes 2, 4, 6, and 8) neuronal cultures were pulse-labeled with ^{35}S-methionine for 5 hr (lanes 1 and 2) or for 1 hr and chased with L-methionine for 2 or 4 hr (lanes 3–8). Full-length and CTF of APLP1 were immunoprecipitated from cell lysates using CT11. Arrow points to the CTF of APLP1.

for 15 min in medium containing ^{35}S-methionine and chased in complete medium for up to 2 hr; TrkB-related polypeptides were immunoprecipiated from detergent lysates using antibody 203 (Ehlers et al., 1995), raised against a conserved peptide domain in the C terminus of all Trk receptors. We document that in PS1$^{+/-}$ cells, the vast majority of immature ~110 kDa TrkB polypeptides (Figure 5A, lane 1) matured to highly glycosylated species of ~140–150 kDa within the 2 hr chase period (Figure 5A, lane 4). On the other hand, in PS1$^{-/-}$ cells, immature TrkB polypeptides persisted throughout the chase period and the levels of highly glycosylated species were significantly diminished (Figure 5A, lanes 5–8). It is not presently clear whether reduced TrkB glycosylation in PS1$^{-/-}$ cells is a reflection of diminished rates of acquisition of mixed oligosaccharide modifications or slower transit of TrkB polypeptides through the secretory pathway. In any event, these studies convincingly demonstrate that in primary cortical neurons, maturation of TrkB receptors is fundamentally altered by the loss of PS1.

Despite the striking reduction in levels of fully glycosylated TrkB in PS1-deficient neurons, it was not inconceivable that underglycosylated TrkB polypeptides might arrive at the surface and still be functional. To test this hypothesis, we examined the autophosphorylation of TrkB receptors after treatment of live PS1$^{+/+}$ and PS1$^{-/-}$ primary neurons with BDNF. Cells were incubated for 10 min with 100 ng/ml BDNF, and total cellular TrkB was immunoprecipitated from detergent lysates using antibody C14, specific for the C-terminal 14 residues of Trk receptors; resulting immunocomplexes were subjected to Western blot analysis with phosphotyrosine-specific antibodies 4G10 and PY99. In contrast to PS1$^{+/+}$ neurons, wherein TrkB receptors undergo robust autophosphorylation after BDNF binding (Figure 5B, lane 2), the levels of autophosphorylated TrkB receptors in PS1$^{-/-}$ neurons are diminished by ~90% (Figure 5B, lane 4; quantified in Figure 5E). The specificity of BDNF-induced tyrosine autophosphorylation of TrkB is shown in Figure 5C; while the general pattern of polypeptides detected with phosphotyrosine antibodies is identical between PS1$^{+/+}$ and PS1$^{-/-}$ neurons, the levels of an ~140 kDa polypeptide, corresponding to tyrosine phosphorylated TrkB, are specifically induced by BDNF treatment (Figure 5C, arrow).

Having demonstrated that PS1 deficiency alters the metabolism of APP, APLP1, and TrkB, we assessed the general effects of loss of PS1 activity on the metabolism of the neuronal secretory apparatus. First, we examined the rate and patterns of appearance of newly synthesized polypeptides on the plasma membrane. Parallel cultures of PS1$^{+/-}$ and PS1$^{-/-}$ neurons were pulse-labeled with ^{35}S-methionine for 15 min, then chased in complete medium for 1 hr. Cell surface polypeptides were modified with biotin and captured with streptavidin. Fractionation of resulting complexes failed to reveal any differences in the patterns of cell-surface molecules between PS1$^{+/-}$ and PS1$^{-/-}$ neurons (data not shown). Our second approach was to pulse-label cells, then chase in complete medium for varying periods of time. The conditioned medium at each time point was fractionated by SDS-PAGE; again, and with very few exceptions, the rates of secretion and accumulation of the vast majority of polypeptides in medium of PS1$^{+/-}$ and PS1$^{-/-}$ cultures were very similar (data not shown).

Discussion

A series of preceding efforts have offered compelling support for the view that FAD-linked mutant PS1 influence APP processing in a manner that leads to increased

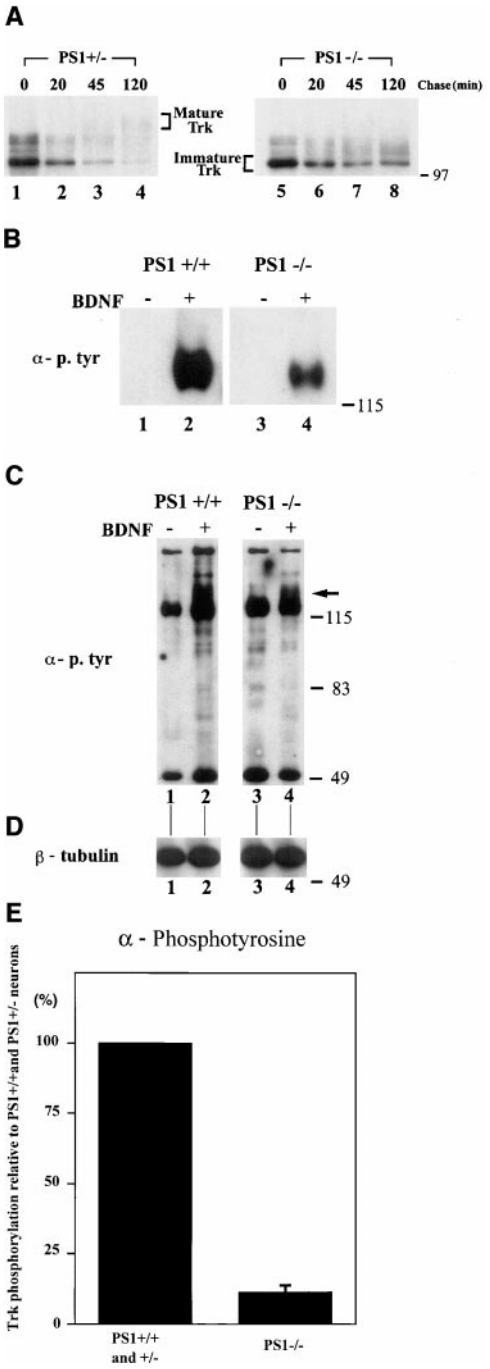

Figure 5. Reduced Levels of Functional TrkB Receptors in PS1$^{-/-}$ Neurons

(A) PS1$^{+/-}$ (lanes 1–4) and PS1$^{-/-}$ (lanes 5–8) neuronal cultures were pulse-labeled with ^{35}S-methionine for 15 min, then chased for 20, 45, and 120 min. Trk polypeptides were immunoprecipitated from detergent lysates using Pantrk203, an antisera that recognizes all Trk receptors. Mature and immature Trk proteins are marked by brackets.

(B and C) PS1$^{+/+}$ (lanes 1 and 2) and PS1$^{-/-}$ (lanes 3 and 4) neuronal cultures were stimulated for 10 min with 100 ng/ml BDNF (lanes 2 and 4) or left unstimulated (lanes 1 and 3). In (B), TrkB polypeptides were immunoprecipitated from detergent lysates with C14, an antisera specific for the C-terminal 14 residues of Trk receptors, and the resultant immunecomplexes were analyzed by immunoblotting with α-phosphotyrosine-specific antibodies 4G10 and PY99. In (C), the supernatant fraction from the C14 immunoprecipitation were immunoblotted with 4G10 and PY99. Arrow points to specific BDNF-induced tyrosine phosphorylated polypeptides.

(D) The blot in (C) was stripped and reprobed using a mouse β-tubulin monoclonal antibody.

(E) Quantitative analysis of BDNF-mediated TrkB autophosphorylation in wt and PS1-deficient neurons. The bar graph depicts the results of three independent experiments. Error bar is SEM.

production of highly amyloidogenic Aβ 1-42/43 peptides (Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996; Citron et al., 1997). More provocative was the discovery that primary neuronal cultures from PS1-deficient mouse embryos fail to secrete Aβ peptides, coincident with intracellular accumulation of APP carboxyl-terminal fragments (CTFs) (De Strooper et al., 1998). These studies were interpreted as proof that PS1 regulates intramembranous γ-secretase processing of APP (Haass and Selkoe, 1998). Despite the novelty of these studies, the specificity of PS1 deficiency was not addressed, a notable omission in view of the paucity of PS1 expression in mammalian cells (Lee et al., 1996; Thinakaran et al., 1996) and the striking developmental deficits in mice lacking PS1 (Shen et al., 1997; Wong et al., 1997).

In the present report, we offer several insights into the role of PS1 on the metabolism and trafficking of a variety of membrane and secretory proteins in primary neurons. Confirming the studies by De Strooper and coworkers, we report that PS1-deficient neurons fail to secrete Aβ. However, and in contrast to the earlier conclusion that accumulated levels of soluble APP derivatives, termed APP$^{s\alpha}$ and APP$^{s\beta}$, in the conditioned medium of primary neurons overexpressing human APP encoded by recombinant Semliki Forest virus (SFV) vectors are not influenced by the loss of PS1 (De Strooper et al., 1998), we document that the rate of appearance of soluble APP (APP$^{s\alpha}$) derivatives in medium of PS1-deficient neurons is markedly increased. In view of abundant evidence that trafficking of overexpressed membrane proteins rarely mimics that of endogenous counterparts, we suggest that any differences in APP trafficking between wt and PS1-deficient neurons would be masked by SFV-driven overexpression.

One of the central questions in the field pertains to the mechanism by which PS1 facilitates "γ-secretase" cleavage of APP. One model, founded on reports that APP and presenilins interact (Weidemann et al., 1997; Xia et al., 1997), proposes that PS1 might serve as an APP "chaperone" to facilitate "γ-secretase" cleavage (De Strooper et al., 1998; Haass and Selkoe, 1998). However, recent studies have failed to confirm the interaction of APP and presenilin either in cultured cells or brain (Thinakaran et al., 1998; P. St. George-Hyslop, personal communication), leading to the alternative proposal that PS1 has a general affect on the turnover of membrane-bound metabolites derived from precursor proteins simply by altering trafficking of these molecules to compartments wherein the elusive "γ-secretases" or other degradative activities are resident. In support of this hypothesis, we report that a CTF derived from an APP homolog, APLP1, also accumulates in PS1-deficient

neurons. Considering the extremely limited homology between the transmembrane domains of APP and APLP1, particularly at the γ-secretase sites, we conclude that PS1 modulates trafficking of residual CTFs derived from APP, APLP1, and perhaps other transmembrane proteins to degradative compartments, rather than acting as a "chaperone" that facilitates intramembrane cleavage by specific proteases. The identities of compartments involved in Aβ production and/or degradation of CTFs have not been determined, but internalization of APP from the cell surface and subsequent recycling appears to be a significant pathway for generating secreted Aβ peptides (Koo and Squazzo, 1994; Koo et al., 1996).

In addition to showing that the metabolism of APP/APLP1 is altered in PS1-deficient neurons, we also report that maturation of the tyrosine kinase receptor, TrkB, is affected by the loss of PS1. Specifically, we demonstrate that the rate of acquisition of complex oligosaccharides by TrkB core proteins is protracted in PS1-deficient neurons. More striking is our demonstration that the levels of BDNF-induced autophosphorylation of TrkB receptors in $PS1^{-/-}$ neurons are nearly eliminated, and we would anticipate that TrkB-activated intracellular signaling events might also be downregulated.

The role of PS1 in modulating trafficking and turnover of selected membrane proteins in neurons is not yet clear. In view of the preponderant steady-state distribution of PS1 in the endoplasmic reticulum (ER) (Cook et al., 1996; Kovacs et al., 1996; Lah et al., 1997), we anticipated that PS1 deficiency might have more global effects on secretory protein metabolism and turnover. However, and despite our demonstration that loss of PS1 alters the metabolism of APP, APLP1, and TrkB, we neither found clear differences in the rate and patterns of appearance of anonymous polypeptides on the plasma membrane of $PS1^{+/-}$ and $PS1^{-/-}$ neurons, nor the rate of secretion of anonymous polypeptides in medium of these cultures. However, it is instructive to note that in yeast lacking membrane proteins, termed Emp24p and Erv25, the rate of delivery of a limited set of soluble proteins from the ER to the Golgi is reduced and some resident ER proteins seem to escape (Schimmoller et al., 1995; Belden and Barlowe, 1996; Elrod-Erickson and Kaiser, 1996). Emp24p and Erv25 reside predominantly in the ER and are incorporated equally into ER-derived vesicles when COPII-coated budding is reconstituted. Very interestingly, reducing the activity of *C. elegans* genes related to Emp24 and Erv25 suppresses certain partial loss-of-function mutations of lin-12/Notch (Chenhui Wen and Iva Greenwald, personal communication). In view of the alterations in protein trafficking/secretion in PS1-deficient neurons, it is not inconceivable that PS1, like Emp24 and Erv25, might be a component of the apparatus involved in selection and sorting of cargo that exits from the ER. Similarly, the inefficient processing of APP and APLP1 CTF, events that occur late in the metabolism of the precursor proteins, might be the result of missorting of specific proteases or, alternatively, accessory components that regulate vesicle budding, targeting, and/or fusion events in the neuronal secretory and/or endocytic pathways.

Experimental Procedures

Primary Cortical Cultures

Cortical neuronal cultures were established from brains of embryonic day 15.5 fetal mice following published protocols (Yavin and Yavin, 1980; Brewer et al., 1993). Briefly, the dissected brain cortexes were suspended in HBSS supplemented with 0.25% trypsin and 0.01% DNaseI and incubated at 37°C for 20 min. The tissues were then transferred to Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and dissociated by repeated trituration. The dispersed cells were collected by centrifugation and plated at $\sim 5 \times 10^5$ cells/35 mm dish (coated with polyethirenimine) in B27/Neurobasal media (GIBCO/BRL). Neurons were allowed to mature for 4–7 days in culture before they were used for experiments.

Infection of Primary Cultures with Recombinant Adenovirus and Metabolic Labeling

APP recombinant adenovirus was prepared via homologous recombination in HEK293 cells using pJM17 and pCA4 vectors (Graham et al., 1977) in which the expressing of wild-type human APP_{695} is under the control of the CMV promoter. The recombinants were expanded several times on HEK293 cells and purified on two successive double cesium chloride gradients. The viral stock was titrated via a plaque assay. Primary neuronal cells cultured for 4 days were infected with 5×10^6 plaque-forming units of adenovirus expressing human APP for 3 days in serum-free medium. For metabolic labeling, neuronal cells were preincubated for 30 min in methionine-free DMEM with 1% dialyzed bovine serum and then labeled with 700 μCi/ml of ^{35}S-methionine in methionine-free medium for 5 hr. For pulse-chase labeling, cells were pulsed for 1 hr (Figure 3) with methionine-free DMEM containing 1 mCi/ml ^{35}S-methionine. Cells were then chased by washing and incubating in DMEM containing 1% dialyzed fetal bovine serum and 1 mM L-methionine at varying intervals, before the cells were lysed in immunoprecipitation buffer containing detergents and a protease inhibitor cocktail (Sisodia et al., 1990).

Determination of Aβ1-42/43 and Aβ1-40 Levels

Two-site ELISAs that specifically detect the C terminus of Aβ were performed to measure Aβ levels as previously described (Suzuki et al., 1994; Iwatsubo et al., 1996). Culture supernatants of neuronal cells infected with adenovirus expressing human APP were collected and analyzed using the BNT77/BA27 and BNT77/BC05 quantitative sandwich ELISA to determine Ab X-42/43 and Ab X-40 levels, respectively.

Immunoprecipitation and Immunoblotting

After metabolic labeling, culture medium and cell extracts were immunoprecipitated as described previously (Sisodia et al., 1990). Immunoprecipitates were fractionated on either 7% or 4%–20% Tris-glycine or 16% Tris-tricine SDS-PAGE. Gels were dried, exposed, and radioactive bands were quantified by phosphorimaging analysis. For immunoblotting analysis, detergent lysates from neuronal cells or fetal mouse brains were prepared as previously described (Davis et al., 1998), and aliquots were fractionated on SDS-PAGE and subjected to immunoblotting with various antisera. Bound primary antibodies were visualized with Protein A-HRP (Sigma, St. Louis, MO) and developed using the Renaissance kit (Dupont/NEN).

TrkB Phosphorylation

TrkB phosphorylation was performed using methods previously described (Riccio et al., 1997). Briefly, neuronal cells were cultured for 6 days, and 100 ng/ml of BDNF was added to cultures for 10 min at 37°C. Cells were then washed in PBS at 4°C, and cell lysates were immunoprecipitated with a Trk C-terminal antibody, C14. Immunoprecipitates were then fractionated on an 8% Tris-glycine SDS-PAGE and immunoblotted with phosphotyrosine-specific monoclonal antibodies, 4G10 and PY99.

Cell Surface Labeling Studies

Cell-surface proteins were biotinylated essentially as described previously (Thinakaran et al., 1996). Briefly, cells were pulse-labeled for

15 min with ^{35}S-methionine, chased for 1 hr in medium containing unlabeled methionine, then placed on ice. Cells were incubated with 0.5 mg/ml NHS-SS-biotin (Pierce) for 30 min, washed with buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 25 mM NH_4Cl, then lysed in immunoprecipitation buffer. Biotinylated proteins were captured using streptavidin-agarose beads (Pierce) and fractionated on SDS-PAGE.

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